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THE STRUCTURE OF SPIROCHAETA NOVYI AS REVEALED BY THE ELECTRON MICROSCOPE^{1, 2}

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The first observations on the morphology and structure of the spirochetes of relapsing fever with the electron microscope were made over three years ago. Ensuing studies showed (1) that with different methods of preparation, the spirochetes exhibited striking variations in morphology, and (2) that many cells possessed structures, such as flagellalike fibers, huge bulges in the organisms, and large, dense granules either attached to the cell or free in the medium, which had not been described by workers using the ordinary microscope. Also some of the spirochetes were fragmented. The specimens for examination were obtained from the blood of infected rats, since cultures of these organisms on laboratory media are unavailable. The spirochetes were repeatedly washed and centrifuged in an attempt to produce satisfactory preparations, and, as a result, parasites having the morphology considered to be normal on the basis of direct examination and stains of infected blood with the usual illumination were rarely present.

In addition, a study of the effect of low temperature on the structure of *Spirochaeta novyi* indicated that a heavy granulation of the protoplasm resulted from the progressive precipitation of a dark-staining phase. In some cases repeated freezing and thawing produced practically complete precipitation and, finally, fragmentation of the spirochetes (Lofgren and Soule, 1945).

It seemed desirable to carry out a more intensive study on the structure of *Spirochaeta novyi* with the electron microscope. The investigation consisted of three parts: (1) the development of a method for the preparation of specimens, (2) the examination of the normal structure of the spirochetes, and (3) an attempt to reveal more of their structural makeup by fragmentation of the cells.

There are no previous reports of electron microscope studies of *Spirochaeta novyi*; however, the structures of other spirochetes have been observed by several workers. Describing Nichol's strain of *Spirochaeta pallida*, Morton and Anderson (1942) stated that "the organisms were frequently surrounded by what appeared to be a slime sheath which occasionally formed thin tendrils projecting from the organisms." They also observed granules, lateral buds, and constrictions in the body of the cell. In a note, Wile, Picard, and Kearney (1942) mentioned that flagellalike processes were seen along the body of the organism but not at the ends of the spirochetes obtained from the primary lesions of syphilis. In addition, a continuous envelope surrounded the cells, and a knoblike struc-

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ture was seen at one end. Definite granules in the protoplasm were described by Mudd, Plevitzky, and Anderson (1942), and "structures which resemble flagella are seen . . . and have been regularly observed in electron micrographs of spirochetes." Later, in a study of the morphology of *Spirochaeta pallida*, *Spirochaeta macrodentium*, and *Spirochaeta microdentium*, the same authors (1943) described a periplast of extreme delicacy which might extend to form a terminal filament. Flagella were frequently present along the sides or near the ends of the cell. Dense granules in the protoplasm were observed in some preparations; also dense, spheroid bodies were attached to the spirochete by a short stock or were free in the medium.

Considerable effort has been directed toward the problem of the preparation of specimens for examination with the electron microscope. The method most widely employed consists of the evaporation of distilled water suspensions of the organisms on collodion films supported by small screens. In most cases, however, it has been necessary to wash and centrifuge the cells in order to free the medium from salts and extraneous matter. "Staining" with the salts of heavy metals (Mudd and Anderson, 1942) and "shadow casting" by the deposition of a thin film of metal on the specimen (Williams and Wyckoff, 1945) have given interesting results.

Spirochaeta novyi, the American strain of the agents of relapsing fever, employed in the following studies, has been maintained in this laboratory for nearly forty years by serial passage in white rats.

EXPERIMENTAL

As indicated earlier, specimens for examination with the electron microscope must be free from extraneous material which might absorb electrons and obscure the objects to be studied. Since the spirochetes were obtained in blood serum suspensions, the substances interfering most with the observations of the specimens were crystals and precipitates of protein, etc. In the present study, a method was developed in which the salts were removed by electro dialysis, resulting in the flocculation of much of the protein and the suspension of the spirochetes in clear fluid. The apparatus constructed for this purpose consisted of a specimen chamber in the center, and at each end an electrode chamber. In actual operation, a collodion sac containing the serum suspension was placed in the center section and distilled water was constantly circulated around it. On either side, coiled glass rods prevented the sac from coming in contact with the plate electrodes. The connections for the electrodes and the circulation of the water were made through rubber stoppers in the ends of the electrode chambers.

The collodion dialysis sacs were prepared according to the method of Novy (1899).

The collodion-covered screens used for mounting the specimens for examination with the electron microscope were prepared by putting two or three drops of U.S.P. collodion on the dust-free surface of double-distilled water in a petri dish. The small screen discs were arranged on the collodion film 2 to 3 mm apart by means of a pair of fine forceps and gently pressed down on the film. This was

continued until an area 1.5 by 4 cm was filled with discs. Then the film and screens were removed by superimposing a clean glass microscope slide on the discs, and, with a needle, the surrounding collodion film was folded around the slide, which was then raised and turned over. The film was allowed to air-dry protected from dust and lint. The discs supporting the dry films were removed by breaking the collodion at the edge of the screens and lifting them off with forceps.

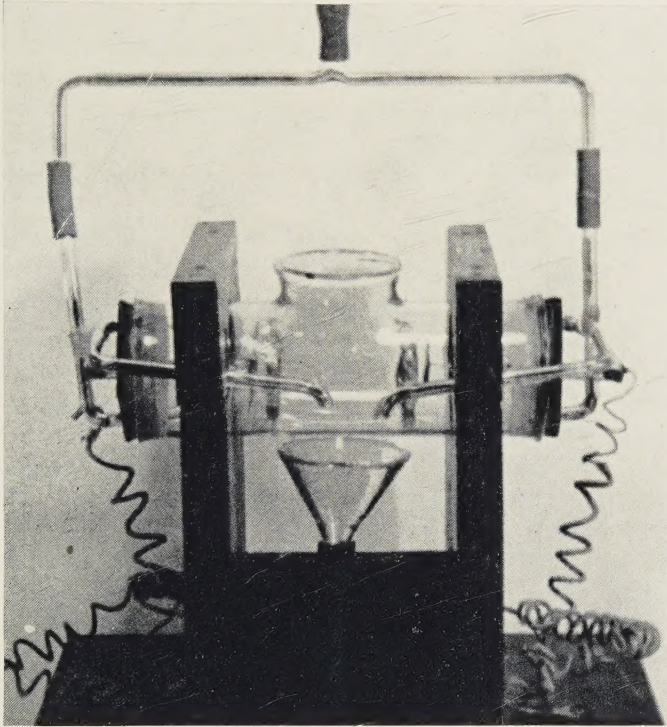


FIG. 1. THE ELECTRODIALYSIS APPARATUS USED FOR THE PREPARATION OF SPECIMENS FOR THE ELECTRON MICROSCOPE

Suspensions of *S. novyi* were obtained by the bleeding of a white rat at the height of the infection, the third day after inoculation. The abdominal and thoracic cavities of the anesthetized animal were opened, and the blood was drawn directly from the heart by means of a glass pipette according to the technique described by Novy and Knapp (1906). The blood was immediately defibrinated by whipping it with a glass rod. After the blood had been centrifuged at 1,700 rpm for 10 minutes, most of the organisms were contained in a white, fluffy layer at the interface between the red blood cells and the serum. This layer was carefully transferred to a collodion dialysis sac by means of a capillary pipette, care being taken not to disturb the cells beneath. The sac was put in the center chamber of the electrodialysis apparatus and the serum suspension

dialyzed for 15 minutes (DC, 180 volts). At the end of this time, minute white flocs had formed in the serum. A small portion of the suspension was placed on a lint-free glass plate on a black background, diluted about 1:50 to 1:75 with distilled water and thoroughly mixed. Then small droplets of the suspension were removed from the clearest areas by means of a small platinum loop and transferred to the collodion films and subsequently examined with the electron microscope. After the first set of films had been prepared, distilled water was added to the suspension to dilute the organisms and mixed thoroughly. Again films were prepared. In this manner, four serial dilutions were made. Examination of the preparations showed that the third or fourth dilution generally gave satisfactory films which contained adequate numbers of organisms. But since there was no way of anticipating the number of organisms present, serial dilutions were made in each instance. It required very little time to check the first few films with the electron microscope. This method has been found to give uniform results.

The organisms prepared in this manner showed uniform, regular spirals, faint, pointed tips, and constricted areas in dividing forms. All the cells appeared more or less uniformly stippled, confirming the data obtained by the use of the ordinary microscope (Novy and Knapp, 1906). (See figure 2, nos. 1 to 7.)

With this information regarding the morphology of the normal organisms, spirochetes were partially disintegrated in an attempt to reveal the basic structure of the cells. Preliminary experiments had demonstrated that repeated centrifugation of the spirochetes of relapsing fever resulted in progressively more severe damage to the cells and finally fragmentation with the complete destruction of the spiral morphology. Suspensions of *S. novyi* were allowed to stand in distilled water to exaggerate the effects of centrifugation due to the deterioration of the organisms.

The spirochetes were obtained and prepared as before. But following electro-dialysis, the suspensions were diluted 1:10 with distilled water, placed in small tubes, 0.4 by 5 cm, and allowed to stand 48 hours. A white, fluffy ring about halfway down the tube contained large masses of spirochetes. A drop of this material was transferred to a lint-free glass plate and diluted about 1:50 to 1:75 with distilled water. The sample was thoroughly mixed and a small drop of the suspension put on each of the collodion films which had been prepared earlier.

An examination of the films made from the suspensions which had been in distilled water for 48 hours revealed considerable variation in the protoplasm of the organisms. Some were dense with no observable differentiation within the cell (figure 3, nos. 1, 2, 3), whereas others showed marked granulation (figure 4, nos. 1, 4). Most of the spirochetes exhibited a definite but undefined variation in the density of the protoplasm. None of the organisms possessed flagella-like fibers attached to the body of the cell; however, some did have a long, rather heavy terminal filament proceeding from one end. Two small forms were sometimes connected by a thin, wavy filament. In most cases, the tips were pointed and less dense than the body of the cell. Spirochetes prepared by this method failed to show a slime sheath.

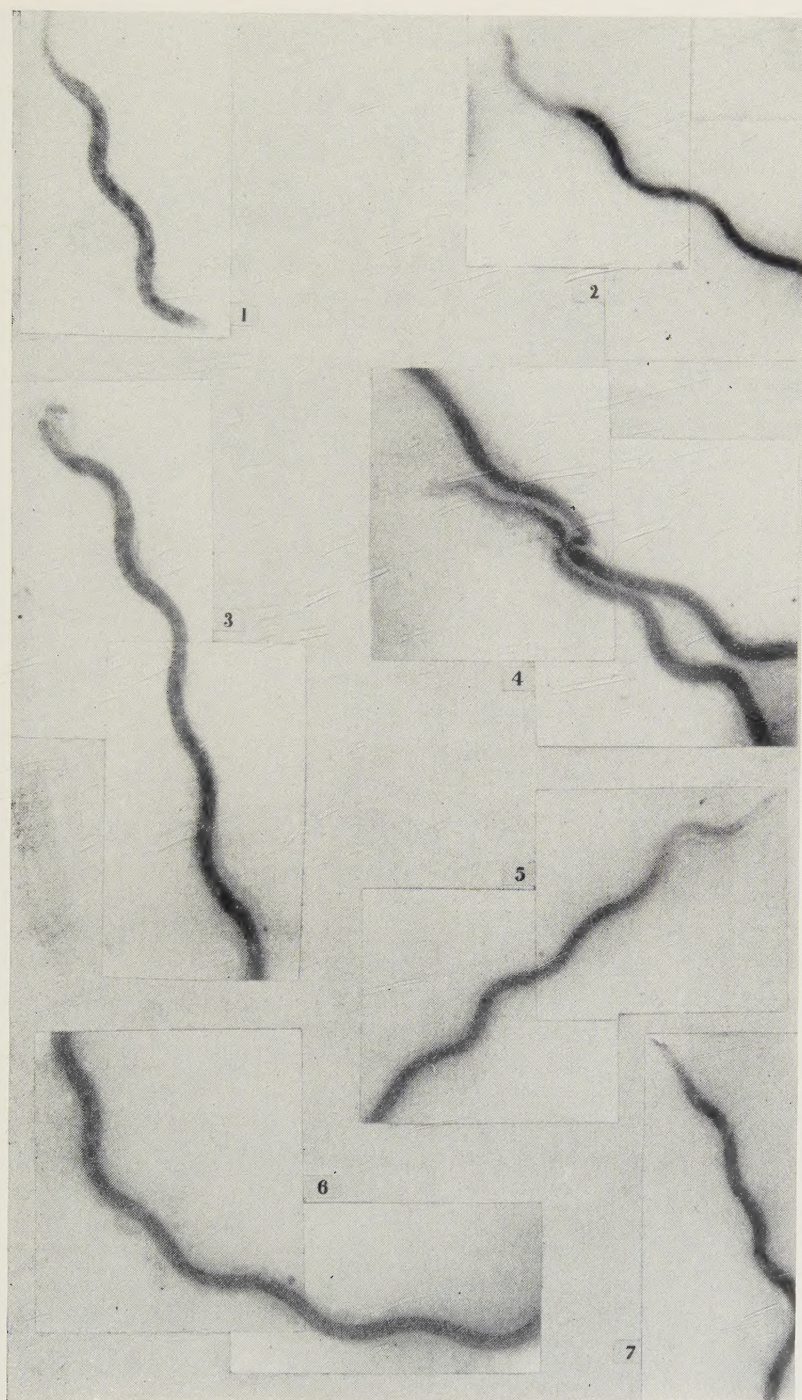


FIG. 2

Nos. 1 to 7. *Spirochaeta novyi* prepared by electro dialysis and dilution (magnification approximately 5,500).

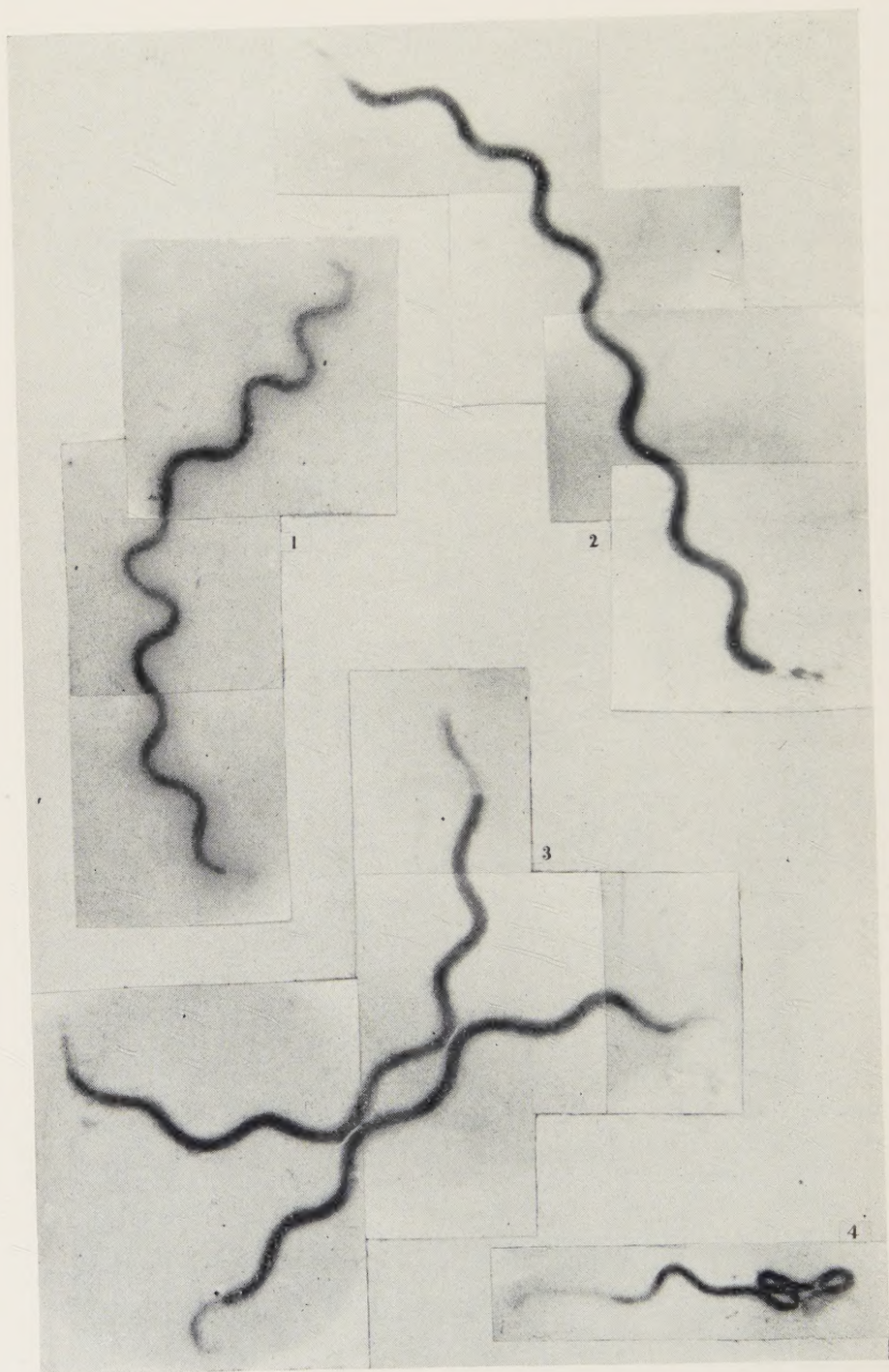


FIG. 3

Nos. 1 to 4. *Spirochaeta noryi* prepared by electro dialysis and dilution, after having stood 48 hours in distilled water (magnification approximately 5,500).

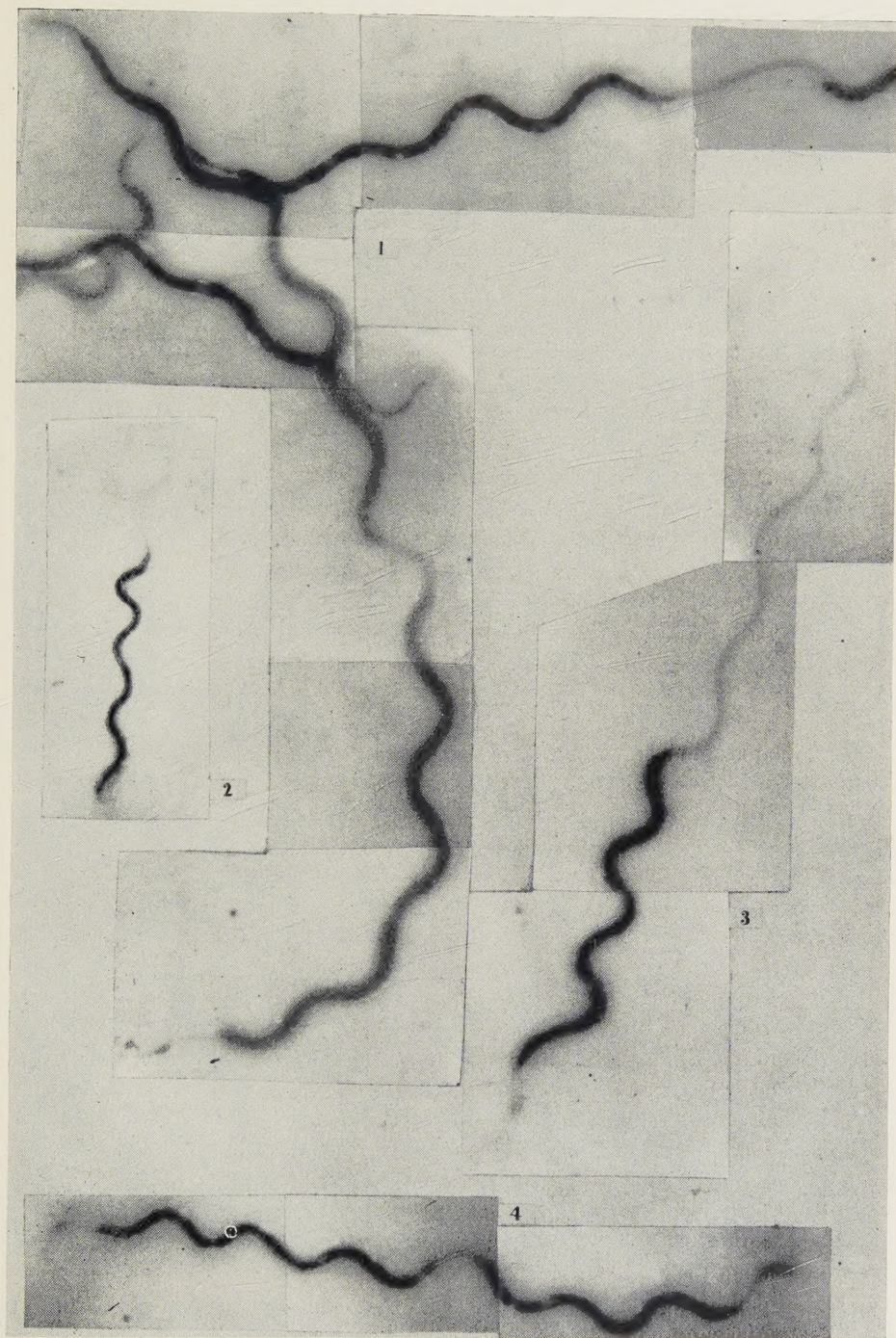


FIG. 4

Nos. 1 to 4. *Spirochaeta novyi* prepared by electro dialysis and dilution, after having stood 48 hours in distilled water (magnification approximately 5,500).



FIG. 5

Nos. 1 and 2. *Spirochaeta novyi* prepared by electro dialysis and dilution, allowed to stand 48 hours in distilled water, and centrifuged three times (magnification approximately 5,500).

No. 3. *Spirochaeta novyi* prepared as above, but centrifuged four times (magnification approximately 5,500).

Nos. 4 and 5. *Spirochaeta novyi* prepared as above, but centrifuged twice (magnification approximately 5,500).

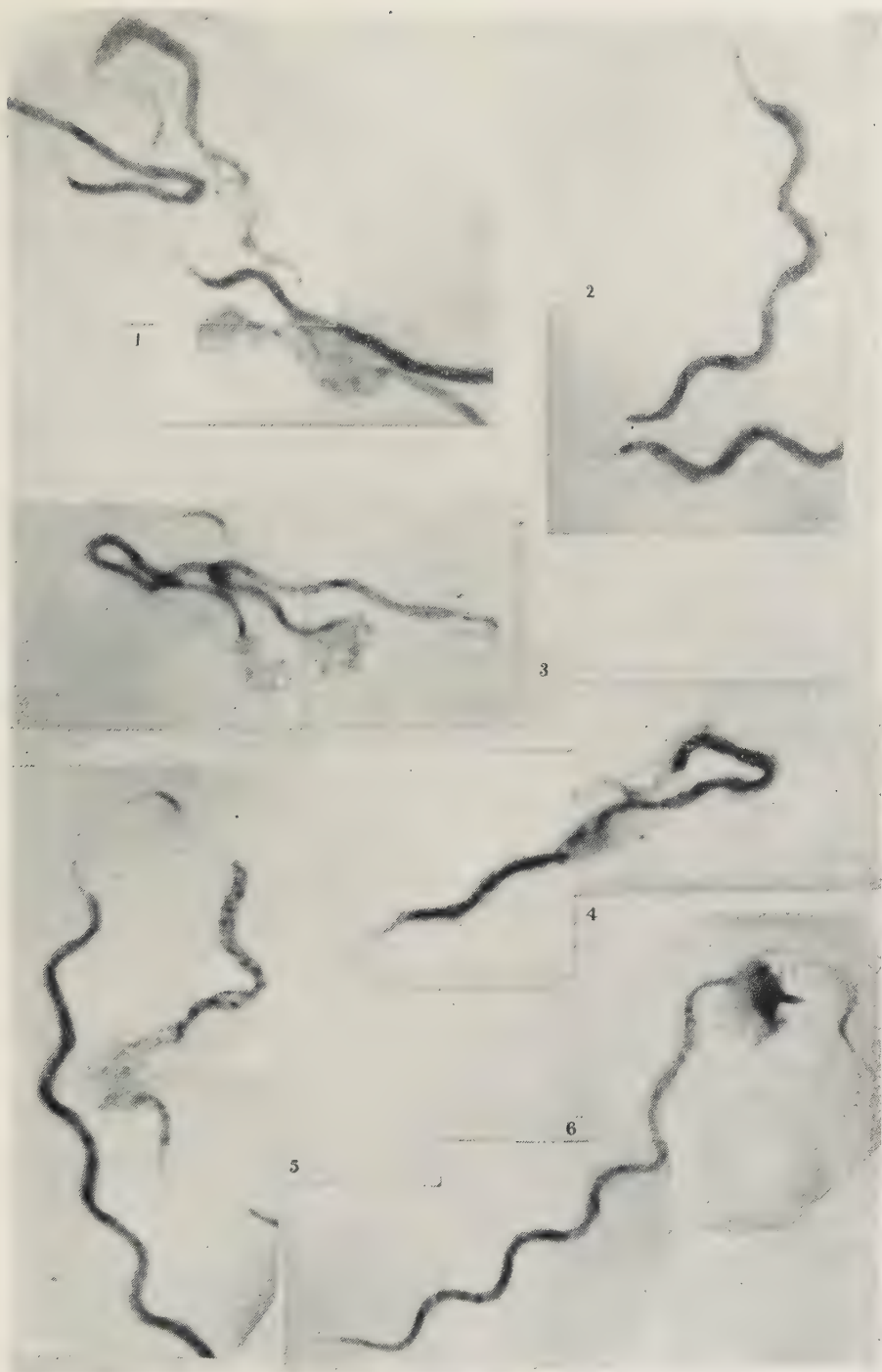


FIG. 6

Nos. 1, 3, 4, 5, and 6. *Spirochaeta novyi* prepared by electrodialysis and dilution, allowed to stand 48 hours in distilled water, and centrifuged four times (magnification approximately 5,500).

No. 2. *Spirochaeta novyi* prepared as above, but centrifuged twice (magnification approximately 5,500).

The remainder of the suspensions which had stood 48 hours in distilled water were transferred to centrifuge tubes and further diluted with distilled water. Following centrifugation at 1,700 rpm for 15 minutes, the supernatant liquid was removed and the sedimented organisms were resuspended as before. This was repeated four times. Films were prepared for examination after the second, third, and fourth washings.

Many of the specimens which had been centrifuged twice showed large granules either attached to the cell or free in the medium (figure 5, no. 5) and fine fibers extending from the body and joining the two portions of the organism (figure 6, no. 2). Following the third centrifugation, most of the spirochetes possessed fine fibers attached to the cell walls; however, some few organisms apparently retained the normal morphology (figures 5, nos. 1, 2). Organisms that had been centrifuged four times all showed marked changes in structure (figure 6, nos. 1, 3, 5).

Most of the organisms which had been subjected to centrifugation showed marked differences from those in figures 3 and 4; however, some of the spirochetes withstood the treatment several times and still seemed to possess their original structure. Flagellalike fibers were often present along the body of the organism, and large, dense granules were occasionally attached to the cell or were free in the field. Many of the spirochetes contained dense protoplasm in which very little differentiation could be seen, whereas others showed marked granulation. The periplast, in some of the forms, no longer enclosed the protoplasm, which had swollen in some areas to twice the original width and become less dense with marked variations in the density, giving the cell contents a distinctly mottled appearance. In this meshlike structure, small, dense granules often occurred at relatively regular intervals. Several organisms were seen in which the swelling had progressed until the protoplasm had spread to form a diffuse but faintly stippled area between the more recognizable portions of the spirochete (figure 6, nos. 1, 3, 4, 5).

Small, dense granules were also seen in these areas. Occasionally a spirochete was found in which the long, thin fibers extended from these regions where the protoplasm had become diffuse.

DISCUSSION AND CONCLUSIONS

In the light of the background of information obtained with the ordinary microscope, the interpretation of the observations made with the electron microscope presents many problems. Differences in density do not necessarily correspond to variations in the staining qualities of the protoplasm of an organism. There are few methods for the preparation of biological material for examination with the electron microscope, and these do not give the consistently excellent results of the techniques for use with the ordinary microscope which are the result of a century of experimentation. At present, the organisms must be suspended in distilled water if reasonably clear backgrounds are to be obtained, and, since many cells are sensitive to changes in osmotic pressure, this may result in swelling or even plasmolysis. The specimens are examined *in vacuo*, thus neces-

sitating dehydration, and without any process of fixation such treatment might be expected to result in some distortion.

In the present study, attempts were made to free the suspensions of organisms from extraneous material by electrodialysis. In most cases there were no conspicuous differences in the morphology of the organisms prepared in this manner and those in fresh blood films, except that many in the films had dried in a random flexion position, whereas the organisms in the water suspensions were more regularly spiraled. Although far greater resolving power was available through the electron microscope, no real addition to the present knowledge concerning the structure of *S. novyi* was made by the examination of normal specimens.

No markedly swollen forms were observed as the result of the suspension of the spirochetes in distilled water for 48 hours. A comparison of the structures of *S. novyi*, before and after centrifugation, indicated that many of the organisms had undergone sufficient deterioration that the treatment resulted in marked swelling, fragmentation, and granulation of the organisms. Long, thin, flagella-like fibers extended from the cell wall, and in some cases seemed to have stretched to two or three times the length of the cell. Some of the spirochetes exhibited a marked granulation of the protoplasm, which progressed beyond that seen in organisms upon standing in serum suspension or even in the latest stages of the disease. Similar large, dense granules had been observed when the spirochetes were subjected to freezing and thawing, the severe breakdown of the protoplasm often resulting in the granules being extruded from the cell as the body of the organism shrank to a thin fiber (Lofgren and Soule, 1945). Centrifugation may have produced a similar breakdown of the protoplasm.

From a study of the structure of *S. novyi* by means of the electron microscope, the organisms appeared to consist of a thin, delicate periplast, which, upon prolonged suspension in distilled water and mechanical damage, was shredded to form long, thin fibers and a colloidal or granular protoplasm. In regions where division was taking place, the cell contents were absent and the connecting membrane was shredded to form many thin fibers joining the two sections. A few organisms showed the presence of a long, rather heavy terminal filament extending from one end of the cell. This was wavy and seemed to be an extension of the periplast. Marked variations in the appearance of the protoplasm of the cells were seen. Some contained very dense granules with accompanying light regions within the cell, whereas others showed no apparent evidence of granulation. Many of the spirochetes were between the two extremes and presented a mottled protoplasm with no clearly defined granules. When the protoplasm had swollen to such an extent that the periplast no longer defined the wall of the cell, a meshlike formation was seen. With more extensive damage, the protoplasm was dispersed to form diffuse, flecked areas. These often contained small, dense, rather evenly spaced granules.

SUMMARY

Suspensions of *Spirochaeta novyi*, obtained from infected rat blood, for examination with the electron microscope were prepared by a process of electro-

dialysis and dilution. Organisms from blood drawn at the height of the infection were regularly spiraled, with smooth cell walls. The protoplasm was either definitely granular or mottled. Most of the cells had pointed tips, less dense than the cell contents; however, a few of the organisms possessed a long, rather heavy terminal filament extending from one end.

The partial fragmentation of the spirochetes following 48 hours in distilled water and repeated centrifugation produced numerous flagellalike fibers extending from the body of the cell and a marked swelling and granulation of the protoplasm. Large, dense granules, either attached to the organism or free in the field, were found in such preparations.

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